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Aqueous two phase extraction for purification of C-phycocyanin

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Abstract

Aqueous two phase extraction is employed for the purification of C-phycocyanin from *Spirulina platensis*. A systematic approach is suggested to arrive at the optimal process parameters of aqueous two phase extraction by considering a case study of C-phycocyanin. The influence of various process parameters such as type of aqueous two phase systems, phase forming salt, molecular weight of the phase forming polymer, system pH, phase composition, phase volume ratio, and type and concentration of neutral salts on differential partitioning of C-phycocyanin is evaluated. Desirable conditions for the purification of C-phycocyanin are found in polymer–salt systems especially in polyethylene glycol (4000)/potassium phosphate system. Increase in purity of C-phycocyanin to 3.52 from initial purity of 1.18 is achieved at pH 6, tie line length of 35.53% with a phase volume ratio of 0.8 in a single step of aqueous two phase extraction. Multiple extractions resulted in further increase in purity of C-phycocyanin without loosing the yield and a maximum purity of 4.05 is achieved in third aqueous two phase extraction. The integration of ultrafiltration with aqueous two phase extraction facilitated the selective removal of polyethylene glycol from the purified C-phycocyanin. Finally, C-phycocyanin is freeze dried to obtain in powder form.

Keywords: Aqueous two phase extraction; C-phycocyanin; Purification; Differential partitioning; Ultrafiltration

1. Introduction

Phycobiliproteins are the major photosynthetic pigments in cyanobacteria. Phycobiliproteins are water-soluble proteins, having covalently attached tetrapyrroles. C-phycocyanin (C-PC) is the major component of the phycobiliprotein family. C-PC exhibits a strong red fluorescence when it is present in native and concentrated form. It is not only used as nutrient ingredient and natural color for food and cosmetics [1] but also used as potential therapeutic agent in oxidative stress-induced diseases [2] and as fluorescent markers in biomedical research [3]. The purity of C-PC is generally evaluated based on the absorbance ratio of A_{620}/A_{280}. C-PC of purity 0.7 is considered as food grade, 3.9 as reactive grade and greater than 4.0 as analytical grade [4]. Various researchers have developed several methods for the purification of C-PC [5–10]. However, almost all these methods of purification of C-PC involve number of steps wherein precipitation, centrifugation, dialysis are used in initial purification, while ion-exchange chromatography and gel filtration chromatography are used in final purification. Major drawback of these protocols is the large number of steps involved, and it is known that higher the number of steps higher is the loss of product yield [11]. Furthermore, the scale-up of these methods is difficult and also expensive. It may be noted that 50–90% of the production cost resides in the purification steps [12]. Hence, there is a need for efficient and economical large-scale bioseparation methods, which will achieve high purity as well as high yield, while maintaining the biological activity of the molecules. One such purification method that meets all these criteria is aqueous two phase extraction (ATPE).

ATPE in many cases offers a better alternative to existing methods, especially in the early processing stages, with regard to scale of operation, low processing time, enrichment of the product and continuous operation for the separation and purification of desired enzymes/proteins from a complex mixture [11,13–16]. In ATPE, selective partitioning of the desired enzymes or proteins to one phase and contaminant proteins to the other phase not only purify the enzymes/proteins but also concentrate them in one of the phases [17,18]. In view of this it was thought prudent to use ATPE for the purification of C-PC. The main objective of the present study is to develop a simple and more efficient downstream process for the purification and concentration of C-PC. More emphasis is given to arrive at the

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optimal process parameters of ATPE by considering a case study of C-PC and is applicable for other biomolecules.

2. Materials and methods

2.1. Chemicals

Polyethylene glycol (PEG) was procured from Sisco Research Laboratories, Mumbai, India. Potassium phosphate, sodium phosphate, ammonium sulphate, magnesium sulphate, sodium sulphate and sodium citrate were procured from Ranbaxy Chemicals, S.A.S. Nagar, India.

2.2. Preparation of crude extract of phycocyanin

Freshly harvested Spirulina biomass (obtained from Department of Plant Cell Biotechnology, CFTRI) was washed with distilled water for about two to three times to remove the culture media components. The biomass was homogenized at a pressure range of 200–400 kg/cm^2 for about 5 min to break the cells and centrifuged at 10,000 rpm for about 10 min to separate the released phycocyanin from cell debris and stored at 4–5 °C and used for the experiments.

2.3. Aqueous two phase extraction

ATPE was carried out by adding predetermined (based on phase diagram from literature, [13,19–22]) weighed quantities of polyethylene glycol and salt to a given quantity of crude extract of C-PC making the total weight of the system 100% on w/w basis. The mixture was stirred thoroughly for about an hour to equilibrate and allowed for phase separation. Concentrations of C-PC and total protein in both the phases were analyzed for estimating the purity and yield of C-PC.

2.3.1. Tie line length (TLL)

$$\mathrm{TLL} = \sqrt{\Delta C_\mathrm{T}^2 + \Delta C_\mathrm{B}^2}$$

where 'ΔC_T' (w/w, %) is the difference in concentration of the predominant top phase forming polymer between top and bottom phases, and 'ΔC_B' is defined similarly for the predominant bottom phase forming component. Concentrations of the phase forming polymer–salts were selected from the literature [13,19–22].

2.3.2. Free volume (V_{FV})

$$V_\mathrm{FV} = \frac{1}{\rho} - \frac{1}{\rho_\mathrm{o}}$$

where 'ρ' is density of one of the phases and 'ρ_0' is density of the respective reference phase solution (blank phase).

2.4. Spectroscopic measurements

Absorption spectra of C-PC were measured using UV–vis spectrophotometer (Double beam spectrometer, Shimadzu, model UV-200S Japan). The ratio of A_{620} to A_{280} gives the purity of C-PC, wherein A_{620} is the maximum absorbance of C-PC and A_{280} is the absorbance of total proteins.

2.5. Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by following the method as described in Methods in Enzymology [23], using 30% polyacrylamide slab gel. Electrophoresis was run at 50 V, 12.5 mA, for about 3–4 h. The gel was stained with a Coomassie Brilliant Blue R250 of 0.05% (w/v), 50% (v/v) methanol and 12% (v/v) acetic acid. The gel was destained using the same buffer without Coomassie Brilliant Blue.

2.6. Ultrafiltration

Ultrafiltration units (Amicon Ultra, 15 ml, 10 and 30 kDa) were procured from Millipore, Bangalore, India and used for the separation of PEG from C-PC.

3. Results and discussion

In aqueous two phase extraction, the purification of most of the proteins is mainly due to the differential partitioning of the target protein to one phase and the contaminant proteins to the other phase. The basis of partitioning of proteins is complex and depends mainly on size, charge and hydrophobicity of the biomolecule. Most of these properties change whenever there is a change in their environment (phase system), which is dependent on many factors such as: (1) type of aqueous two phase system; (2) phase forming salt; (3) molecular weight of the phase forming polymer; (4) pH of the system; (5) phase volume ratio; (6) type and concentration of neutral salts. Due to the multiple factors, the selection of suitable phase system is a search in a multidimensional space requiring a number of experiments to achieve optimization efficiently. If each of these factors were analyzed at least for three different values, over 2000 ($3^7 = 2187$) experiments would be necessary [24]. The optimized condition thus obtained is suitable only for a given biomolecule and does not hold good for the other biomolecule.

A properly designed systematic approach is required in order to reduce this large number of experiments. Selection of key parameters is an important task and requires thorough knowledge of solute to be studied. In view of this, experiments were planned through a systematic approach as shown in Fig. 1, wherein all the parameters, that are expected to affect the partitioning of proteins and enhance the purification of C-PC, are considered in a logical sequence. Details of selection of each of these parameters and their effect on protein partitioning have been discussed in the following sections.

3.1. Selection of type of aqueous two phase system

In order to know the suitable type of phase system (polymer–polymer or polymer–salt), both the polymer–polymer (PEG/maltodextrin) and polymer–salt (PEG/potassium phosphate) phase systems were prepared using crude extract of